Attenuation of MSC Hypertrophy in 3D Culture via Treatment with a Retinoic Acid Receptor Inverse Agonist

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Introduction: Engineered cartilage constructs based on mesenchymal stem cells (MSCs) have developed to the point where cartilage-like materials can be grown in vitro from a variety of material formulations [1,2,3]. For instance, we have shown that hyaluronic acid (HA) hydrogels support MSC chondrogenesis, with near-native tissue properties achieved when constructs are seeded at high densities and cultured for long durations [4,5]. Despite this promise, concerns remain regarding MSC phenotypic stability after chondrogenesis. Originally described by Peltarri and co-workers [6], and since reported by numerous laboratories [7,8,9], chondrogenic MSCs have an unfortunate tendency to undergo hypertrophy when removed from pro-chondrogenic conditions and implanted subcutaneously, or when chondrogenically differentiated MSCs are cultured in a specialized hypertrophic induction media [8,10]. This hypertrophic induction can likewise instigate MSC hypertrophy and mineralization in HA, with variables such as hydrogel stiffness and density and dynamic loading promoting or preventing hypertrophic conversion, respectively [11,12]. Hypertrophic transition by MSCs is not by surprising, given their close homology to transient ‘chondrocytes’ that make up the growth plate in developing animals [13,14]. Retinoic acid (RA) signaling plays a key role in growth plate hypertrophy, and quite interestingly, treatment with RA receptor (RAR) agonists attenuates heterotopic ossification in vivo induced by BMP2 implantation and muscle injury into skeletally mature animals [15]. Interestingly, RAR inverse agonists (IA) (that is, compounds that block RAR function by recruitment of corepressors) promote chondrogenesis when provided early in culture [16]. Given these findings, we hypothesized that treatment of MSC-seeded constructs with RAR IA, in the context of hypertrophic challenge after chondrogenesis, might delay or reverse hypertrophy and/or mineralization. To test this hypothesis, we cultured bovine MSCs in HA hydrogels and challenged them with hypertrophic induction.

Methods: Passage 2 juvenile bovine MSCs were seeded at 60x106 cells/ml in methacyrlated 1% HA hydrogel. Cylindrical constructs (2mm height x 4mm diameter) were cored from the gel slab after photopolymerization [17]. Constructs were pre-differentiated in a chemically defined medium containing 10ng/mL TGF-ß (CM+) for 14 days. After this, constructs were cultured for an additional 21 days under 8 conditions: CM+, CM- (w/out TGF), hypertrophic medium (Hyp; CM- with 1nM T3, withdrawal of dexamethasone and no TGF-ß) and osteogenic medium (Ost; hypertrophic with beta-glycerolphosphate, BGP). Each of these first four conditions was additionally supplemented with the RA receptor (RAR) inverse agonist BMS493 (BMS) at 2mM. On day 35, constructs were assessed for mechanics (by unconfined compression testing), GAG and DNA content (DMMB and PicoGreen assays). Cartilage matrix and mineralization was assessed by histology (Alcian blue, Picrosirius red, Alizarin red, and H&E staining). MSC apoptosis was determined by TUNEL assay in histological sections. Statistical differences between groups were determined by one-way ANOVA with Bonferroni’s post hoc testing.

Results: Consistent with our previous findings, hypertrophic induction of MSCs in HA hydrogels was apparent by day 35. Alcian blue staining of proteoglycans was highest in control groups, but markedly reduced in both the Hyp and Ost groups. Furthermore, in Ost medium, regions of calcification were apparent via Alizarin red staining (Fig. 3). Mechanical analysis on day 35 showed a significant decline in equilibrium modulus (p<0.01: Hyp vs. CM+, Ost vs. CM-, p<0.001: Hyp vs. CM-, p<0.05: Ost vs CM+) in hypertrophic and osteogenic groups compared to CM+ and CM- groups (Fig. 2). GAG content followed a similar trend, with lower content in both the Hyp and Ost groups. Treatment with BMS during hypertrophic conversion attenuated some changes in construct properties and appearance. For instance, the mechanical properties (p<0.05 for Hyp group) (Fig. 2) and GAG content (for Hyp and Ost groups) were higher with BMS treatment compared to Hyp controls (p<0.05). BMS treatment resulted in smaller cells in Hyp conditions, and higher proteoglycan and collagen content in every condition (Fig. 1). Likewise, staining for apoptosis was reduced in the Ost-group with BMS treatment compared to Ost controls (Fig. 3).

Discussion: In this study, we validated that MSC-seeded HA hydrogels coupled with hypertrophic media challenge can produce features of hypertrophic conversion. Further supplementation of hypertrophic medium with a phosphate source (BGP) led to marked mineral deposition within the hydrogel. This hypertrophic conversion resulted in a reduction in construct mechanical properties and GAG content, and increased cell size. Treatment with BGP also increased the level of apoptosis in the construct. However, inclusion of the RAR inverse agonist BMS493 attenuated these hypertrophic changes. Specifically, when BMS was concurrently administered along with hypertrophic or osteogenic medium, a decrease in cell size, increase in construct mechanics and GAG content, and decrease in mineralization and apoptosis were observed. Based on these findings, current
studies are exploring the timing and dose of BMS to most efficaciously prevent hypertrophic changes, as well as the duration of its effect and delivery in a therapeutic setting. Further, we are investigating the pathways through which BMS493 exerts its effects.

**Significance:** The RAR inverse agonist BMS493 can attenuate hypertrophic conversion of MSC seeded constructs, and may be useful in producing stable engineered tissue for cartilage regeneration.

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**References:**

![Fig.1: Histological analysis (Alcian blue staining) of MSC-seeded HA constructs under control and hypertrophic conditions and with BMS treatment; scale bar 200μm.](image)
Fig. 2: Mechanical properties and GAG content of MSC-seeded HA constructs with hypertrophic challenge (top row) and with BMS treatment and hypertrophic challenge (bottom row).
Fig. 3: Mineralization (top) and apoptosis (bottom) on day 35 is reduced with BMS treatment of MSC-seeded constructs that were challenged with Ost medium. Upper row: Alizarin red stain, scale bar 1mm; lower row: TUNEL stain (green apoptotic cells) with DAPI counterstain, scale bar 500µm.

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